Alterations in melatonin and serotonin signalling in the colonic mucosa of mice with dextran-sodium sulfate-induced colitis

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Abbreviations: DSS, dextran sodium sulphate; 5-HT, serotonin; SERT, serotonin transporter; trinitrobenzene sulfonic acid; TNBS, 5-hydroxytryptophan;5-HTP, 5-hydroxyindoleacetic acid; 5-HIAA, gastrointestinal; GI, inflammatory bowel disease; IBD, boron-doped diamond; BDD, sodium deoxycholic acid; DCA, High performance liquid chromatography; HPLC, disodium ethylene-diamine-tetra-acetate; EDTA, N-acetyltransferase; NAT, hydroxyindole-O-methyltransferase; HIOMT, tryptophan hydroxylase 1; TpH1, aromatic L-amino acid decarboxylase; L-AADC.

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Abstract

Background and Purpose: Inflammatory bowel disease (IBD) is characterized by pain, bleeding, cramping and altered gastrointestinal (GI) function. Changes in mucosal serotonin (5-HT) signalling occur in animal models of colitis and in humans suffering from IBD. Melatonin is co-released with 5-HT from the mucosa and has a wide variety of actions in the GI tract. Here we examined how melatonin signalling is affected by colitis and determined how this relates to 5-HT signalling. **Experimental Approach:** Using electroanalytical approaches, we investigated how 5-HT release, reuptake and availability as well as melatonin availability are altered in dextran sodium sulfate (DSS)-induced colitis in mice. Studies were conducted to explore if melatonin treatment during active colitis could reduce the severity of colitis. **Key Results:** We observed an increase in 5-HT and a decrease in melatonin availability in DSS-induced colitis. Significant reduction in 5-HT reuptake was observed in DSS-induced colitis animals. A reduction in the content of 5-HT was observed, but no difference in tryptophan levels were observed. A reduction in deoxycholic acid stimulated 5-HT availability and a significant reduction in mechanically stimulated 5-HT and melatonin availability was observed in DSS-induced colitis. Orally or rectally administrated melatonin once colitis was established did not significantly suppress inflammation. **Conclusion and Implications:** Our data suggest that DSS-induced colitis results in a reduction in melatonin availability and an increase in 5-HT availability, due to a reduction/loss of tryptophan hydroxylase 1 enzyme, 5-HT content, and serotonin transporters. Mechanosensory release was more susceptible to inflammation when compared to chemosensory release.

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Introduction

Ulcerative colitis is one of the two major forms of inflammatory bowel disease (IBD) and is characterized by inflammatory damage of the intestinal tissue which results in symptoms of pain, bleeding, cramping and altered gastrointestinal (GI) motility and secretion (Bassotti et al., 2014). The inflammatory damage in ulcerative colitis is largely restricted to the mucosal epithelium of the colonic wall. The mucosal epithelium contains enterochromaffin (EC) cells, which release transmitters following either chemical or mechanical stimulation (Racke et al., 1995; Racke & Schworer, 1991). EC cells serve as epithelial transducers between the lumen and the underlying neuronal and glial network of the enteric nervous system (Bertrand & Bertrand, 2010; Mawe & Hoffman, 2013). Various studies have shown that EC cells act as a key regulator of propulsive motility patterns (Heredia et al., 2013; Lavoie et al., 2015; Morris et al., 2016; Patel, 2016). The EC cell has been shown to release a range of chemical transmitters, of which most studies have focused on serotonin (5-HT) (Costedio et al., 2007; Mawe & Hoffman, 2013). However, few studies have focused on melatonin (Bubenik, 2001; Raikhlin, 1976), which has been shown to be expressed and released from the mucosa (Bubenik et al., 1977; Patel, 2008; Raikhlin et al., 1976; Raikhlin et al., 1975), and is a metabolite of 5-HT.

Human and animal studies have shown that inflammation results in changes to various aspects of mucosal 5-HT signalling (Costedio et al., 2007). Using the trinitrobenzene sulfonic acid (TNBS)-induced model of colitis, changes in 5-HT content, the number of EC cells, the amount of 5-HT released and expression of the serotonin transporter (SERT) have been observed (Linden et al., 2003; Linden et al., 2005; O'Hara et al., 2004). Dextran sodium sulfate (DSS) has also been utilised to induce colitis. DSS colitis is a mixed Th1/Th2 cytokine mediated colitis and is generally less severe than TNBS colitis. In murine DSS colitis, 5-HT availability increased primarily by an increase in the numbers of EC cells and/or of content of 5-HT in these cells (Bertrand et al., 2010a). In humans with ulcerative colitis, there were decreases in storage of 5-HT and EC cells, no change in 5-HT release and a loss in SERT function (Coates et al., 2004). The observations in animal models and in humans with colitis are somewhat inconsistent, due in part to the differences between IBD and chemical models, however, in virtually all conditions and models examined to date, there is an increased 5-HT availability in the inflamed gut (Costedio et al., 2007).

Melatonin has been shown to have a wide range of effects on GI function (Bubenik, 1999; Bubenik, 2002). In particular, melatonin is believed to function as a physiological antagonist of the actions of 5-HT (Bubenik & Pang, 1994). Melatonin release has not been examined in colitis, however, increased expression of melatonin synthesis enzyme hydroxyindole-O-methyltransferase has been observed in patients with ulcerative colitis (Chojnacki et al., 2013). Changes in melatonin signalling have been observed with ageing in mice (Diss et al., 2013). Of interest, melatonin treatment has been shown to reduce markers of inflammation in mouse colon and the severity of DSS colitis in mice, due to its action as an anti-oxidant (Bertrand et al., 2010b; Pascua et al., 2011; Pozo et al., 2010). A study have also indicated that adjuvant melatonin treatment may help in sustaining remission in patients with ulcerative colitis (Chojnacki et al., 2011; Mozaffari & Abdollahi, 2011).

Here we have examined changes in 5-HT and melatonin mucosal signalling following DSS colitis in mice. We have utilised electrochemical sensors as they provide temporally and spatially precise measurements of changes in release, availability and reuptake from live tissue. Using such approaches, we have monitored how the 5-HT and melatonin signalling mechanism are altered following chemical or mechanical stimulation. We have also utilised chromatography to monitor the changes in precursors and metabolites to provide insight into key enzymes involved in the mucosal signalling processes. Finally, we explored if oral or rectal administration of melatonin during active colitis could reduce the severity of disease.

Methods

Animals. Wild-type male CD1 mice (6-8 weeks; 28 ± 2 g, Charles River, Montreal, QC) were used. Animal protocols were approved by the University of Calgary Animal Care Committee, and were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Animal studies were also reported in compliance with ARRIVE guidelines (Kilkenny et al., 2010; McGrath & Lilley, 2015). Animals were housed in polystyrene cages with free access to food and tap water and maintained on a 12h light-dark cycle in a temperature and humidity controlled room. All animals were killed by cervical dislocation under deep isofluorane anaesthesia. The group sizes for untreated and treated animals were not identical due to the natural variation in the severity of colitis and therefore a greater number of colitis animals were utilised to see correlations between signalling and tissue damage.

Colitis. Colitis was induced in 5-6week old male CD1 using dextran sodium sulfate (DSS; molecular weight 36,000-50,000; MP Biomedical, Solon, OH; 5% weight/volume in water) dissolved in drinking water for 5 days, followed by 2 days of normal drinking water. DSS-treated animals were euthanized 7 days after initiation of treatment. No adverse events were observed. Body weight, stool consistency and the presence of fecal blood was monitored throughout. The extent of damage induced by intestinal inflammation was assessed using a modified version of an established macroscopic damage score (Cluny et al., 2010; MacEachern et al., 2015). Briefly, the total colonic damage score consisted of the sum of a score for the change in body weight (percentage body weight loss from initial where 1=0-5%, 2=5.1-10%, 3=10.1-15% and 4>15%), a score for the change in colon length (percent of control colon length where 1=75-85%, 2=65-74.9% and 3<64.9%; control colon length=9.2 cm), the percentage of the colon inflamed, summed with scores for the presence or absence of erythema (0 or 1), diarrhoea (0 or 1; defined as unformed fecal pellets) and fecal blood (0 or 1).

Dosing studies. Treatment of mice began 4 days following initiation of DSS during active colitis. Mice received either vehicle (0.5% DMSO in water) or melatonin (8mg/kg; Sigma M5250) once daily by gavage via a 30g feeding needle (3cm; Fine Science Tools, Vancouver, BC, Canada) or by enema via a 5 French flexible feeding tube (Covidien, Mansfield, MA, USA) inserted 4.5cm intrarecatally, under isofluorane anesthesia. A volume of 200 µl/30g body weight was administered. In order to determine if any effects of melatonin were specific to it's action at the melatonin receptor a group of mice receiving both oral and rectal melatonin were also treated with Luzindole, a specific melatonin receptor antagonist. Mice received either vehicle (0.5% DMSO) or Luzindole (5mg/kg; Tocris 0877) ip 15 minutes prior to the gavage or enema treatment in a volume of 200 µl/30g body weight. Mice were killed 7 days after initiation of DSS. We observed one death in the oral melatonin, oral melatonin plus luzindole and rectal melatonin groups during the study. The extent of damage induced by intestinal inflammation was assessed by an individual blinded to the treatment group.

Amperometric approach curve profiling for measuring 5-HT release and reuptake. For approach curve profiling measurements, constant potential amperometry was utilised. A platinum wire was used as the auxiliary electrode and a "no leak" Ag|AgCl electrode (3 M KCl, Cypress Systems Inc., USA) served as the reference electrode in a three-system configuration.

The working electrode consisted of a 76 µm boron-doped diamond (BDD) microelectrode. All the electrodes were placed in a flow bath that was continuously perfused with warm (37°C) oxygenated (95% O_2 and 5% CO_2) Krebs' buffer solution, pH 7.4 (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose) at a flow rate of 4 ml min^{-1}. 1 cm^2 Colon segments taken 2 cm proximal to the rectum were placed into the Krebs' buffer solution prior to measurements. The segments were bisected along the mesenteric border to open up the preparations and the tissue was pinned down with the mucosal layer upmost. Tissues were allowed to rest for 15 minutes prior to commencing recordings.

For measurements, the BDD microelectrode was affixed to a micromanipulator (Fine Scientific Tools, USA) and placed > 5 millimetres over the centre of a tissue piece in the bulk of the media. The BDD electrode was held at a potential of +650 mV *vs.* Ag|AgCl. Measurements were carried out using a previously published protocol (Marcelli & Patel, 2010; Zhao et al., 2010). Briefly, the electrode was carefully positioned over the tissue for a duration of 40 s for each of the following electrode – tissue distances: 1000, 800, 600, 400 and 200 µm (see Supplementary Figure 1A). Measurements were repeated 2 times on each tissue segment. Following this the tissue was perfused with 1 µM fluoxetine for 30 minutes prior to repeating the measurement protocol.

Constant-potential amperometry detection of 5-HT and melatonin availability during mechanical and chemical stimulation. For the detection of 5-HT and melatonin a three-electrode system was utilised where the BDD electrode served as the working electrode. Isolated colonic tissue segments were and mounted on the flow bath and constantly perfused with oxygenated warm Krebs buffer. Measurement of 5-HT and melatonin availability was conducted as previously described (Patel, 2008). Briefly, the BDD electrode was placed in bulk media and then placed over the tissue at an electrode tissue distance of 500 µm using a manipulator for a duration of 40 s. Following this the electrode was retracted back to bulk media. Sequential measurements from the same tissue location were carried out at +650 mV for detection of 5-HT and +800 mV for the detection of melatonin and 5-HT. These measurements were repeated 5 times over various regions of the tissue section.

The protocol was slightly modified for mechanical stimulation, where the BDD electrode was taken from bulk media to an electrode-tissue distance of 500 µm. For the first 40 s the sensor

was utilised to monitor basal levels of the transmitters. Following this without any movement of the BDD electrode, a glass capillary was utilised to gently touch the mucosal villi located 200 µm adjacent of the BDD electrode for 20 s. After the mechanical stimulation was removed, a further 40 s recording was carried out prior to retraction of the BDD electrode into bulk media (Supplementary Figure 1B). To understand the effect of chemical stimulation the tissue perfused with 10 µM sodium deoxycholic acid (DCA) for 20 minutes and the same protocol as above was conducted.

High-performance liquid chromatography measurements High performance liquid chromatography (HPLC) was utilised to monitor the levels of 5-HT precursors and metabolites was previously described (Chau & Patel, 2009; Parmar et al., 2011). Briefly, a 1 cm^2 segment of the distal colon was isolated and using a sharp scalpel, the mucosal layer of the tissue was scraped. The mucosal tissue was placed in 500 µl of ice cold 0.1 M perchloric acid. Samples were homogenised and centrifuged at 14,680 g for 10 minutes at 4 °C. Prior to chromatographic analysis samples were filtered through a 0.2 µm Phenex RC membrane syringe filter.

The HPLC apparatus consisted of a Jasco HPLC pump (Model: PU-980) and Rheodyne manual injector equipped with a 20 µl loop. A Kinetic® ODS 2.6 µm 100 mm x 2.1 mm i.d. analytical column with a guard column (Phenomenex®, Macclesfield, UK) was employed. The HPLC system was run at a flow rate of 0.1 mL min^{-1}. CHI630B potentiostat (CH Instruments, Austin, TX, USA) was used to control the detector voltage and record the current. A 3-mm glassy carbon electrode (flow cell, BAS) served as the working electrode and was used with a Ag|AgCl reference electrode and a stainless-steel block as the auxiliary electrode. Amperometric recordings were carried out, where the working electrode was set at a potential of +850 mV vs. Ag|AgCl reference electrode. Control and data collection/processing were handled through the CHI630B software. The stock buffer for the mobile phase was comprised of the following: 0.1 M sodium acetate, 0.1 M citric acid and 27 µM disodium ethylene-diamine-tetra-acetate (EDTA). This was then buffered to pH 3.0. The mobile phase was prepared with the stock buffer mixed with methanol in the ratio of 8:2 (v/v) and degassed after mixing.

Standard solutions were prepared from 1 mM stock standards of each analyte and were made up in 0.1 M perchloric acid (BDH). Each of the standard solutions were prepared on the day of

analysis and stored at 4°C prior to injection. A calibration plot was obtained for each 5-HT precursors and metabolite. The peak areas obtained from chromatographic analysis of all injected samples were converted to concentrations utilising the calibration responses. The concentration of each 5-HT precursors and metabolite was normalized by protein content using the Bradford method to remove any variation in amount of tissue sample. To obtain the activity of key intracellular enzymes the ratios of 5-hydroxytryptophan (5-HTP):tryptophan and 5-HT:5-HTP were obtained.

Data interpretation and statistics. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). During the generation of data, the severity of the colitis score was blinded from the experimental data. For electrochemistry measurement it was not possible to blind the untreated to treated animals for colitis due to the visual nature of the tissue damage, however this was randomised and blinded for HPLC analysis. For analysis of the amperometric approach curve profiling traces, the current was recorded at each of the electrode-tissue distances and plotted against the electrode-tissue distance. This resultant plot generally shows the response of an exponential decay. The natural log of the current in whole numbers (pA scale) was calculated and plotted against the electrode-tissue distance and a linear regression line was fitted to the data enabling the slope and intercept the vertical axis to be derived. From the slope and intercept, information on the re-uptake and release of 5-HT can be obtained, respectively as previously shown (Marcelli & Patel, 2010). For amperometric measurement of 5-HT and melatonin, the difference in the current over the tissue compared to baseline was obtained. During mechanical stimulation, the current difference over the tissue to that during mechanical stimulation was measured. To obtain the current response for melatonin, the response obtained at +800 mV was subtracted from that obtained at +650 mV as previously shown (Diss et al., 2013; Patel, 2008). In both amperometry approach curve profiling and basic amperometry measurements, the current for 5-HT and melatonin were converted to concentration using calibration plots. For comparison between control measurements and those in the presence of colitis, data were presented as mean ± 95 % confidence interval and were compared using two-way ANOVA followed by post-hoc pair-wise comparisons with Bonferroni's test when F achieved $P<0.05$ and there was no significant inhomogeneity. For all data, $P<.05$ was accepted as a level of statistically significant difference (analysed using Graphpad Prism 6.07, GraphPad Software Inc.). Correlation plots were also obtained by plotting individual animal responses of the various parameters investigated against the macroscopic damage score.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2017), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

Results

Changes in the availability of 5-HT and melatonin in DSS colitis

There was a significantly greater amount of melatonin availability when compared to serotonin availability ($P<0.05$, Figure 1A). A significant increase in 5-HT availability and a significant decrease in melatonin availability was observed from DSS-treated animals when compared to untreated mice ($P<0.05$, Figure 1A). The ratio of melatonin:5-HT significantly decreased from 2.1 ± 0.1 in untreated animals to 0.3 ± 0.2 in DSS-treated animals ($P<0.05$, Mann-Whitney test, Figure 1B). Correlations of the macroscopic damage score with 5-HT availability were obtained, where no meaningful relationship was observed ($P=0.281$, Figure 1C). A significant decrease in melatonin availability was associated with increasing macroscopic damage scores ($P<0.05$, Figure 1D).

Alterations in 5-HT release and reuptake

Measurements at varying distances between the tissue and electrode provides the means to understand changes in release and reuptake. Figure 2A shows the natural log (Ln) current obtained from wildtype animals in the presence and absence of 1 µM fluoxetine, which is known to inhibit SERT. The slope, which is a marker of reuptake is less steep in presence of 1 µM fluoxetine, suggesting inhibited reuptake. Ln current responses from wildtype and DSS-induced mice are shown in Figure 2B, where changes in the slope can be observed.

Measurement of the intercept provided insight into the changes with 5-HT release. There was a significant increase in 5-HT release in wildtype animals in the presence of fluoxetine ($P<0.05$) and in DSS-induced mice under control conditions ($P<0.05$, Figure 2C). There was

no difference in 5-HT release in wildtype tissues in the presence of fluoxetine when compared to DSS-treated animals in either absence or presence of fluoxetine (Figure 2C). Measurement of the slope provided insight into the changes in reuptake between untreated and DSS-treated animals. When studies were conducted in fluoxetine, there was significant decrease in the rate of reuptake in wildtype animals (P<0.05) when compared to control. There was a significant decrease in the rate of reuptake in DSS-treated animals when compared to wildtype animals under control conditions (P<0.05, Figure 2D). In the presence of fluoxetine, there was a significant decrease in the rate of reuptake in DSS-induced animal colonic tissues (P<0.05, Figure 2D) when compared to control.

Correlations of 5-HT release levels measured in the presence of fluoxetine against macroscopic damage score from individual animals treated with DSS are shown in Figure 2E. The correlation was conducted using data where release was monitored in the presence of fluoxetine to remove the bias of release due to reuptake at the epithelium surface. There was a significant decrease in 5-HT release in the presence of fluoxetine with increased macroscopic damage score (P<0.05, Figure 2E). The changes in 5-HT release at macroscopic damage scores between 5-8 were minor, however significant reductions in 5-HT release are observed with macroscopic damage score $\geq$ 8.5. There was a significant decrease in the rate of 5-HT reuptake with increasing macroscopic damage score (P<0.05, Figure 2F).

Chromatographic measurements of 5-HT precursors and metabolites

Chromatographic measurements of colonic mucosal tissue were conducted, where no significant differences in the levels of tryptophan were observed between untreated and DSS-treated animals (Figure 3A). Significant decreases in 5-HTP (P<0.05, Figure 3B), 5-HT (P<0.05, Figure 3C) and 5-hydroxyindoleacetic acid (5-HIAA, P<0.05, Figure 3D) were observed in DSS-induced colitis mice when compared to untreated mice. Decreases in the ratio of 5-HTP:tryptophan were observed in colonic tissues of animals treated with DSS when compared to untreated animals (P<0.05, Mann-Whitney test, Figure 3E). No significant changes in the 5-HT:5-HTP ratio were observed in DSS treated animals when compared to untreated animals (Figure 3F). Using the amperometric and chromatographic data, the ratio of intracellular to extracellular 5-HT can be obtained. Significant decrease in the intracellular to extracellular 5-HT was observed in DSS treated animals (P<0.05, Supplementary Figure 2).

Influence of mechanical and chemical stimulation on 5-HT and melatonin availability in DSS colitis

Studies were conducted to understand the influence of DCA to stimulate 5-HT release in DSS colitis. Figure 4A shows the basal release of 5-HT, due to low level mechanical stimulation by the flow of the physiological media within the bath, where there is a significant increase in 5-HT availability in DSS treated animals (P<0.05, n=9-16). The contribution of DCA to drive 5-HT availability was shown to be of the same capacity of that observed at basal stimulation. This 5-HT availability contribution induced by DCA was significantly lower in DSS-inflamed colonic tissue when compared to wildtype (P<0.05, Figure 4A).

A glass capillary was utilised to evoke an additional mechanical response above the basal stimulation. Mechanical stimulation in wildtype animals results in approximately half the response under basal stimulation. In untreated and DSS induced inflammation tissue, the component of additional 5-HT availability was greater due to DCA stimulate when compared to mechanical stimulation (P<0.05). There was a reduction in the 5-HT availability contribution by mechanical stimulation (P<0.05) and combined mechanical-DCA stimulation (P<0.05) in DSS induced animals when compared to wildtype (Figure 4A).

There was a significant reduction in the DCA stimulated 5-HT availability with increasing macroscopic damage score (P<0.05, Figure 4B). No correlation was observed with mechanically stimulated 5-HT availability and macroscopic damage score (Figure 4C).

Similar studies were conducted with melatonin, where under basal stimulation, there was a significant reduction in melatonin availability in DSS-induced animals (P<0.05, Figure 4D). Under chemical stimulation with DCA, there was no additional contribution to the underlying basal response. No differences in the levels of melatonin availability due to DCA activation were observed between untreated and DSS-treated animals. In untreated tissue, the component of additional melatonin availability was greater due to mechanical stimulation when compared to DCA stimulation (P<0.05). Additional mechanical stimulation elevated the melatonin availability by half the response of the basal measurement (Figure 4D). A significant reduction in mechanically stimulated melatonin availability was observed in DSS treated animals when compared to wildtype (P<0.05), which resulted in a significant reduction in melatonin

availability in DSS treated animals when a combined DCA and mechanical stimulation was utilised ($P<0.05$, Figure 4D).

No correlations were observed between DCA (Figure 4E) and mechanically (Figure 4F) stimulated melatonin availability and macroscopic damage score.

Investigation of the impact of orally and rectally delivered melatonin on DSS-induced colitis in mice

Studies were conducted to explore if treatment with melatonin could reduce the severity of inflammation in DSS-induce colitis in mice. Animals were treated both orally and rectally. No differences in body weight during the 7 days post DSS initiation were observed when treated with melatonin or luzindole plus melatonin orally compared to vehicle control (Figure 5A). No significant difference in the total damage score between the melatonin and melatonin plus luzindole treatment groups were observed when compared to the vehicle control group when treated orally (Figure 5B). No differences in body weight during the 7 days post DSS initiation were observed when treated with melatonin or luzindole plus melatonin rectally when compared to vehicle control (Figure 5C). No significant difference in the total damage score between the melatonin and melatonin plus luzindole treatment groups were observed with compared to the vehicle control group when treated rectally (Figure 5D).

Discussion

The main findings of this study are that 5-HT availability is increased and melatonin availability is decreased in a mouse model of DSS-induced colitis. Such increases in 5-HT availability are supportive of a previous study that utilised electrochemical methods to monitor 5-HT in DSS-induced colitis in mice (Bertrand et al., 2010a).

How does 5-HT availability increase and melatonin availability decrease in DSS-colitis?

The increased 5-HT availability was evaluated using an electrochemical approach that monitored levels at various distances from the tissue surface. This provides the ability to gain insight into 5-HT release and reuptake (Marcelli & Patel, 2010). Our findings showed that

reuptake was significantly reduced in DSS-induced animals, to a similar extent that could be achieved in untreated tissues that were exposed to 1 μM fluoxetine. This suggests that SERT activity is lost and/or impaired secondary to inflammation induced by DSS. This was supported by reduced levels of 5-HIAA observed in mucosal tissue using chromatography. Loss and/or impairment of SERT is a consistent finding in all animal and human studies that have investigated changes in 5-HT signalling process during inflammation (Costedio et al., 2007).

In measurements in physiological buffer, we observed a significant increase in 5-HT release in DSS-induced animals when compared to wildtype, however this doesn't provide a true reflection of release, as in wildtype animals, the reuptake process by SERT is active, which can reduce the signal observed at the electrode, providing an underestimation of release. Therefore, it is most logical to understand how release is altered in the presence of SERT inhibitor fluoxetine, so a reflective contribution of release can be monitored. Our study indicates that release is not altered in either untreated and DSS-treated animal tissues that were exposed to fluoxetine. To further support this, no differences were observed in the release of 5-HT in DSS-induced animals when compared to wildtype tissue in the presence of fluoxetine. This finding supports observations obtained in TNBS-induced colitis in mice and in human patients with ulcerative colitis (Coates et al., 2004; Linden et al., 2005).

We observed no changes in tryptophan levels, but significant decreases in 5-HTP and 5-HT levels in mucosal tissue of DSS-treated animals when compared to untreated animals. These changes suggest that 5-HT content or the number of EC cells are reduced following inflammation. A significant reduction in the 5-HTP:tryptophan ratio was observed, suggesting that the tryptophan hydroxylase 1 (TpH1) enzyme is impaired and/or lost as a result of inflammation. No changes were observed in 5-HT:5-HTP, suggested that the aromatic L-amino acid decarboxylase (L-AADC) enzyme is not altered during inflammation. Our findings are contradictory to other published findings, which in both TBNS and DSS-induced inflammation in animal models have observed increases in either 5-HT content or number of EC cells (Bertrand et al., 2010a; Linden et al., 2003; Linden et al., 2005; O'Hara et al., 2004). This may be due to the nature of the studies conducted as increased content was thought to be due to increases in the number of EC cells monitored extracellularly, whereas we have monitored intracellular 5-HT levels. However, our findings agree with observations in human patients with ulcerative colitis, where a reduction in 5-HT content was seen (Coates et al., 2004).

Our study is the first to show that melatonin levels were significantly reduced following inflammation. Melatonin has been shown to be synthesised on demand from 5-HT by the enzymes N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT) (Bubenik, 2001). Therefore, the reduction in the amount of melatonin produced is directly associated with reduction in 5-HT.

Relationship between 5-HT and melatonin changes with inflammation

Our study is the first to show that ratio of melatonin:5-HT availability decreases from approximately 2 to 0.5 following inflammation. This is most likely due to the loss in SERT function and a reduction in 5-HT content. This reduction in the melatonin:5-HT content is most likely to have implications to the function of the colon, as the relationship between these two molecules have been shown to direct motility (Bubenik, 1986; Bubenik & Dhanvantari, 1989; Thor et al., 2007). However, these molecules also have an interesting ying-yang relationship with regards to the regulation of inflammation. Various studies have shown that elevated 5-HT can induce inflammation and exacerbate colitis (Bischoff et al., 2009; Ghia et al., 2009). By contrast melatonin is well known to act as an anti-oxidant and reduce markers of inflammation (Pascua et al., 2011; Pentney & Bubenik, 1995; Talero et al., 2014). Our findings provide important insight as they are suggestive that in wildtype animals, a greater amount of melatonin compared to 5-HT availability is present, which may be responsible for providing resilience of the colon to react to inflammation. Following DSS treatment, this trend is reversed and therefore this will most likely exacerbate the inflammation, as 5-HT acts to induce this and melatonin cannot serve to reduce this. Preserving the balance in the ratio of 5-HT and melatonin may be key to reducing the severity of the inflammation in colitis. These changes are suggestive that adjuvant therapy using melatonin may be of benefit in restoring the ratio of 5-HT and melatonin, which may explain observations shown in studies that have led to sustaining remission using melatonin treatment (Chojnacki et al., 2011).

Alterations in chemo and mechano-stimulated 5-HT and melatonin release

5-HT is well known to be released following mechanical and chemical stimulation of the mucosal epithelium. However, little is known about what drives melatonin production other than that it is produced on demand. Within our study, we utilised DCA as a chemical stimulant

to drive 5-HT release. DCA has been shown to increase 5-HT secretion through transport into the EC cell to induce ERK phosphorylation (Braun et al., 2007). A glass capillary was utilised to distort the villus and therefore activate mechnosensory ion channels, such as transient receptor potential channels to drive 5-HT release (Nozawa et al., 2009; Patel, 2016).

Within our studies, we observed that there was a reduction in both DCA and mechanical stimulated 5-HT availability in DSS-induced colitis animals, of which the reduction was of a greater extent following mechanical stimulation. This would suggest that chemosensory channels are more resilient than mechanosensory ion channels in the inflamed state.

No detectable melatonin availability was observed with DCA, suggesting that DCA does not influence or drive melatonin production. However, like 5-HT availability, a significant reduction in melatonin availability was observed in DSS-induced colitis animals following mechanical stimulation. This suggests that activation of mechanosensory ion channels can enhance the production of melatonin, which is most likely through protein kinase A which has been shown to phosphorylate NAT (Rosiak & Zawilska, 2005).

Does oral or rectally delivered melatonin suppress inflammation in DSS-induced colitis in mice?

Within our study we utilised both oral and rectal delivery of melatonin, in which neither showed the ability to significantly reduce the total damage score and therefore the severity of colitis. However, we did see a modest but not significant reduction in damage score when melatonin treatment was given as an enema.

Our study is at odds with other studies that have seen improvement in either TNBS or DSS induced colitis when treated with melatonin. However, with most of this studies melatonin was given before the onset of the disease (Cuzzocrea et al., 2001; Liu et al., 2017; Trivedi & Jena, 2013; Zielińska et al., 2016), which likely accounts for the differences observed when compared to our results where treatment was given once symptoms were present. Studies also observed phenotypic changes such as shortening of the colon to an extent greater that DSS alone (Zielińska et al., 2016), which from our observations is highly correlated with a worsening of colitis.

Although our data were not significant, there are still indications that melatonin could play a role as an adjunctive therapy, however the route of administration, the duration of treatment and dose needs significant further investigation.

Are the changes in 5-HT and melatonin correlated with the extent of damage by inflammation?

There is considerable interest in utilising chemical signalling molecules as potential biomarkers for bowel conditions (Clarke et al., 2009), however, the majority of studies have looked for changes without showing any correlative information on the extent of the condition and changes in mucosal signalling. Our study is the first to show the relationship between 5-HT and melatonin signalling with the extent of damage in colitis.

We observed that no correlation was present between macroscopic damage score and 5-HT availability, however, a correlation was present with melatonin availability. When the availability of 5-HT was investigated for contribution of reuptake and release, there was a significant decrease in reuptake with increased damage score. However, for 5-HT release, no changes in 5-HT release were observed until a very high macroscopic damage score of 8 of which afterwards there was a significant decline in 5-HT release. These changes suggest that 5-HT availability alone may not be a good marker of the extent of damage, but melatonin may be more reflective. It also indicates that 5-HT reuptake by SERT is less resilient to inflammation than processes that regulate 5-HT release, which are lost only after much higher macroscopic damage score.

We also noticed that increased 5-HT availability due to DCA stimulation was lost with increasing macroscopic damage score, however, the mechanically stimulated 5-HT and melatonin availability was lost over the entire range of damage scores. This indicates that mechnosensory ion channels are high susceptible to inflammation when compared to chemosensory receptors. Therefore, chemosensory receptors may be a better target to alter 5-HT signalling in colitis. However, this study only provides reflection on one chemosensory target and others may be more or less susceptible during inflammation.

The observed changes with DSS-induced inflammation on 5-HT and melatonin signalling are summarized in Figure 6.

Conclusion

This study provides insight into the changes in 5-HT and melatonin signalling in DSS-induced colitis in mice. We observed decreases in 5-HT content, a reduction in TpH1 activity and a reduction in SERT, which resulted in increased 5-HT availability. Due to the reduction in 5-HT content, we observed a reduction in melatonin availability. The loss in melatonin availability correlated with increasing extent of damage. This would suggest that adjuvant therapy with melatonin may be of benefit but further investigation of route and timing of treatment is necessary. Mechanosensory ion channels were lost with inflammation, however, DCA stimulated 5-HT availability was lost with increasing extent of inflammation. Overall these findings provide novel insights into changes in mucosal signalling with inflammation and such approaches can provide valuable tools in understanding functional changes observed in inflammatory bowel disease.

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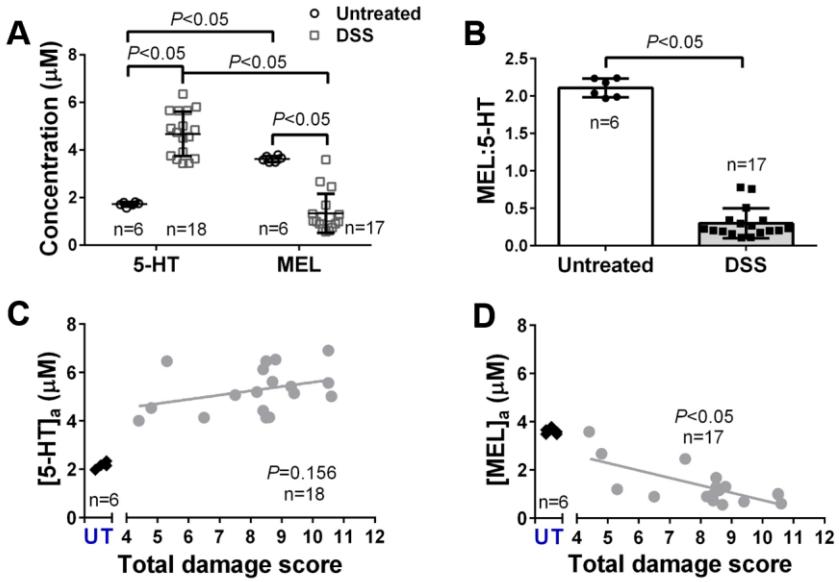

Figure 1. Alterations in 5-HT and melatonin availability in animals with DSS induced colitis. (A) Current responses of measured 5-HT and melatonin availability in wildtype and DSS inflamed isolated colonic segments. (B) Ratio of melatonin:5-HT. (C) shows correlations of 5-HT and (C) melatonin availability versus total damage score. The mean damage score in DSS treated mice was 8.2 ± 0.9. Data shown as mean ± 95% C.I.

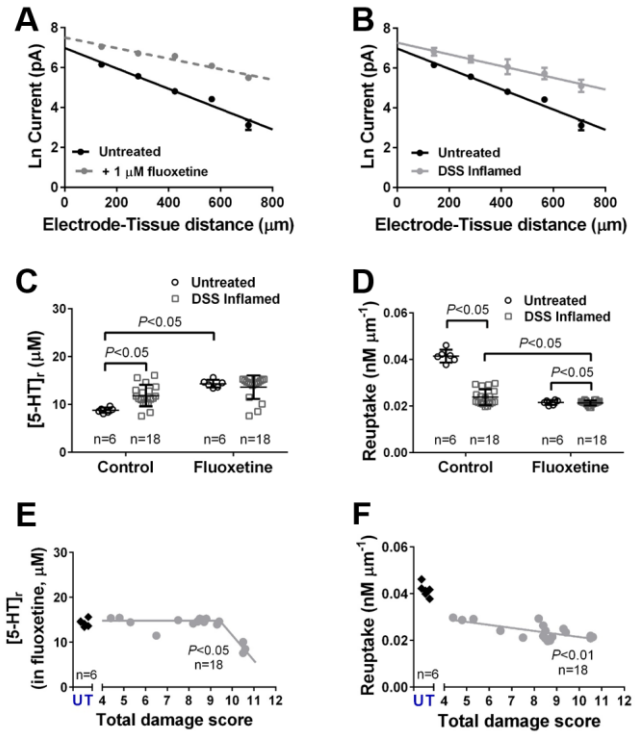

Figure 2. Changes in 5-HT release and reuptake following inflammation. Ln current response at varying electrode-tissue distances obtained from amperometry approach curve profiling when investigating the (A) influence of fluoxetine on wildtype colonic tissue and (B) effect of DSS-induced inflammation. (C) Release of 5-HT. (D) Reuptake of 5-HT. Correlation of (E) 5-HT release and (F) 5-HT reuptake against total damage score. Data shown as mean ± 95% C.I.

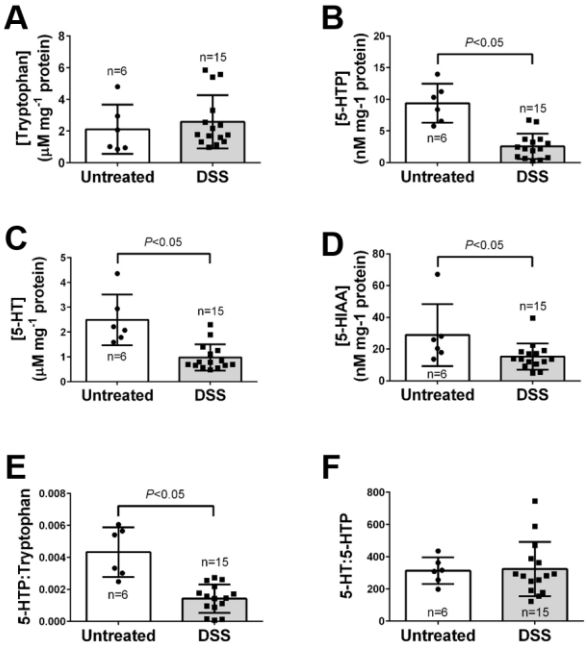

Figure 3. Changes in precursors and metabolites involved in 5-HT signalling process. Changes in levels of (A) tryptophan, (B) 5-HTP, (C) 5-HT and (D) 5-HT before and after DSS-induced inflammation. Ratios of (E) 5-HTP:tryptophan and (F) 5-HT:5-HTP. Data shown as mean ± 95% C.I.

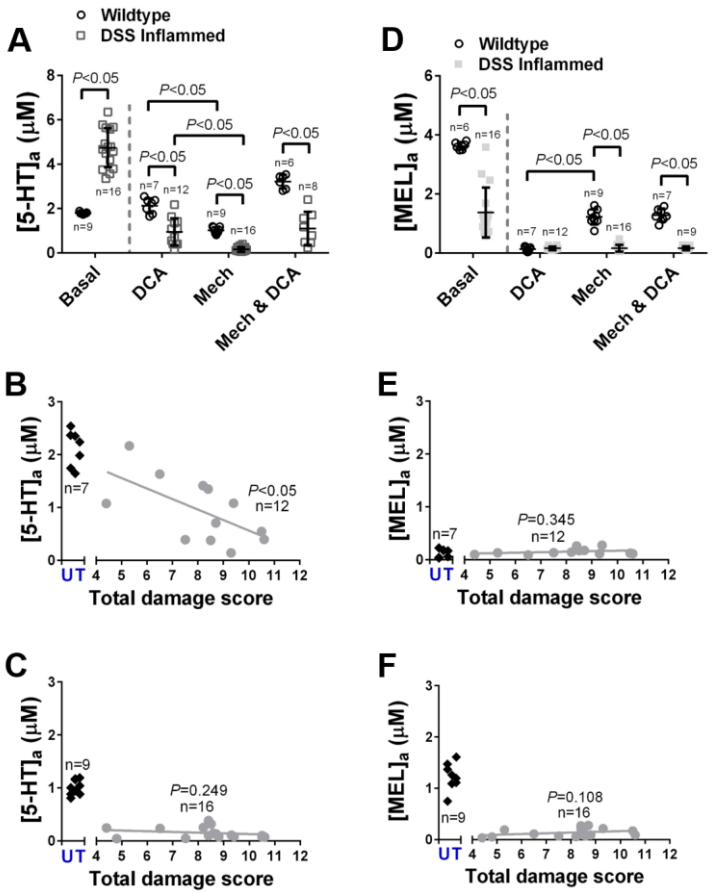

Figure 4. Contribution of 5-HT and melatonin availability following DSS-induced inflammation. Contribution of DCA and additional mechanical stimulation on (A) 5-HT and (D) melatonin availability. Correlations of 5-HT availability due to (B) chemical and (C) additional mechanical stimulation against total damage score. Correlations of melatonin availability due to (E) chemical and (F) additional mechanical stimulation against macroscopic damage score. Data shown as mean ± 95% C.I.

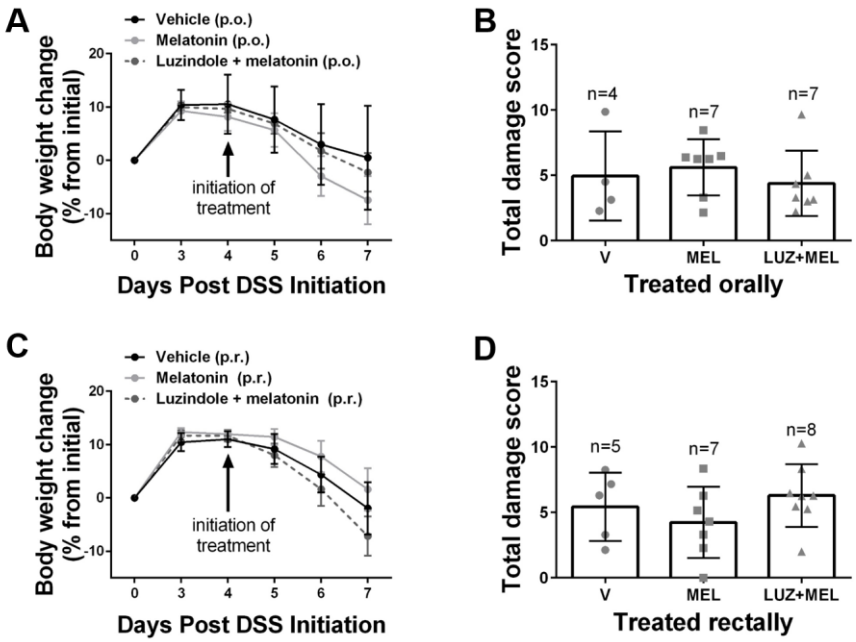

Figure 5. Effect of orally and rectally delivered melatonin on parameters of colitis. (A) Change in body weight during progression of colitis using DSS, where oral (p.o.) treatment was initiated on day 4. (B) The resultant total damage score for orally dosed melatonin (MEL) against the vehicle (V) and melatonin plus luzindole (MEL + LUZ). (C) Change in body weight during induction of colitis using DSS, where rectal (p.r.) treatment was initiated on day 4. (B) The resultant total damage score for rectally administered melatonin (MEL) against the vehicle (V) and melatonin plus luzindole (MEL + LUZ). Data shown as mean ± 95% C.I.

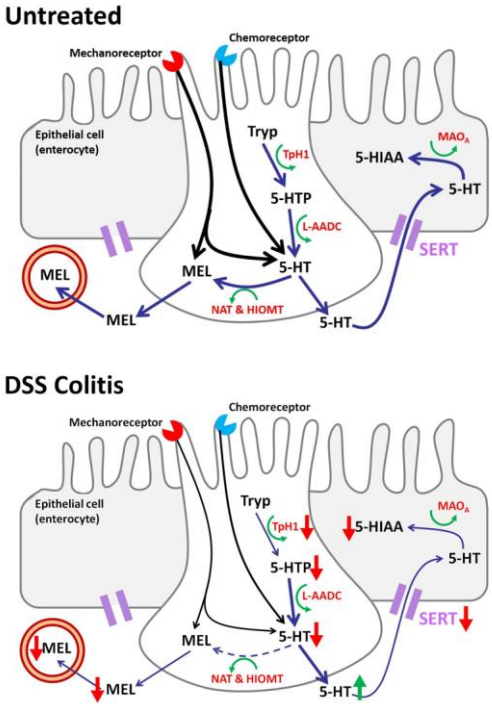

Figure 6. Changes in 5-HT and melatonin signalling process following DSS-induced colitis. Where decreased in intracellular 5-HT result in reduction in melatonin availability, whilst increased in 5-HT availability are observed due to a loss/impairment in SERT. Where Tryp is tryptophan; 5-HTP is 5-hydroxytryptophan; 5-HT is serotonin; MEL is melatonin; 5-HIAA is 5-hydroxyindoleacetic acid; TpH1 is tryptophan hydroxylase 1; L-AADC is aromatic L-amino acid decarboxylase; NAT is N-acetyltransferase; HIOMT is hydroxyindole-O-methyltransferase; MOA_A is monoamine oxidase A; SERT is serotonin transporter.

Table of links

TARGETS		
GPCRs	**Transporters**	**Transporters**
MT1	TPH-1	SERT (SLC6A4)
MT2	AADC	
	MAO-A	

LIGANDS	
fluoxetine	melatonin
deoxycholic acid	luzindole